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Proliferation

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13. ABSTRACT (Maximum 200 Words) The mechanism of inhibition of cancer cell proliferation by vitamin A is poorly understood because many of the targets that mediate the retinoid-dependent growth suppression are not known. We have recently identified a novel retinoid-responsive gene target, <b>TIG3</b> , that we believe may be a key player in mediating the retinoid-dependent suppression of tumor cell proliferation. Understanding the mechanism of TIG3 action may provide insights that lead to innovative new anti-breast cancer therapies. Exploiting this potential requires that we understand how TIG3 inhibits cell proliferation – the major goal of this proposal. <b>Specific Aim 1</b> Subcellular location plays a major role in determining function, and knowing location provides clues about function. Therefore, our first goal is to localize TIG3 in breast cancer cells. <b>Specific Aim 2</b> Most proteins contain distinct functional domains, some responsible for localization and others for function. We hypothesize that TIG3 is divided into distinct functional domains that are important for growth suppression. We are testing this hypothesis. <b>Specific Aim 3</b> The TIG3 amino acid sequence/structure reveals no obvious catalytic functional domains, suggesting that TIG3 acts by modulating the function of other proteins. A major goal of the study is to identify these targets. We have 1) constructed a plasmid-based TIG3 expression systems and used it to express TIG3 in cells, 2) identified a perinuclear localization of TIG3 in cells, 3) demonstrated that the TIG3 carboxy-terminal hydrophobic domain guides appropriate subcellular localization, 4) shown that the TIG3 carboxy-terminal tail is required for optimal cell killing, 5) constructed an adenovirus expression system that permits efficient TIG3 expression for biochemical studies, and 6) we identified type I transglutaminase as a TIG3 target in cells.				
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## INTRODUCTION

The mechanism whereby vitamin A analogs inhibit breast cancer cell proliferation is not known. We have identified a retinoid-regulated gene, **TIG3**, that we believe may be a key mediator of retinoid-dependent suppression of breast tumor cell growth. Vitamin A treatment increases TIG3 mRNA levels in breast tumor cells, and this is correlated with growth suppression, suggesting that TIG3 may mediate the retinoid-dependent suppression. Understanding how TIG3 works may lead to innovative new anti-breast cancer therapies. Exploiting this potential requires that we understand how TIG3 inhibits cell proliferation - the major goal of this proposal. **Specific Aim 1** The goal of this aim is to localize TIG3 subcellular distribution. We have localized TIG3 distribution to the perinuclear region and shown that it localizes in membrane structures via its carboxyl terminus. **Specific Aim 2** We hypothesize that TIG3 is divided into distinct functional domains that are important for growth suppression. We have tested a variety of mutants and have identified critical regulatory domains. **Specific Aim 3** The TIG3 amino acid sequence/structure reveals no obvious catalytic functional domains, suggesting that TIG3 acts by modulating the function of other proteins. The goal of this specific aim is to identify candidate target proteins using co-immunoprecipitation, affinity chromatography and two-hybrid screening. We had made substantial progress on *Specific Aims 1 and 2* at the time of the past report. This information remains, essentially intact below. Substantial new progress has been made regarding *Specific Aim 3*. This is presented towards the end of this summary. Together this information summarizes the achievements during the entire support period.

## BODY

### Specific Aims 1 & 2

- *Subcellular location plays a major role in determining function, and knowing location provides clues about function. Therefore, our first goal is to use immunological and cell fractionation methods to localize TIG3 in cells.*
- *Most proteins contain distinct functional domains, some responsible for localization and others for function. We hypothesize that TIG3 is divided into distinct functional domains that are important for growth suppression. To test this, we will construct a series of mutants and measure the ability of each mutant to suppress growth using a breast cancer cell colony formation assay.*

We will describe the construction of our TIG3 mutants, their detection in cells, and their subcellular distribution, and their bioactivity in this section. One manuscript has been published based on this work.

Construction of TIG3 inducible adenovirus expression constructs TIG3 is an 18 kDa 164 amino acid growth suppressor protein that is present in very low levels in cells. An important goal of this study is to identify the role of various functional domains within the protein and to determine which domain controls subcellular localization. The sequence of TIG3 is shown in **Fig. 1**.

10	20	30	40	50
MASPHQEPKP	GDLEIEIFGRLG	YEHWALYIGD	GYVIHLAPPS	EYPGAGSSSV
60	70	80	90	100
FSVLSNASEV	KRGRLEDVVG	GCCYRVNNSL	DHEYQPRPVE	VISSAKEMV
110	120	130	140	150
GQKMKYSVS	RNCEHFVAQL	RYGKSRCKQV	EKAKVEVGVA	TALGILWAG
160				
CSFAIRRYQK	KATA			

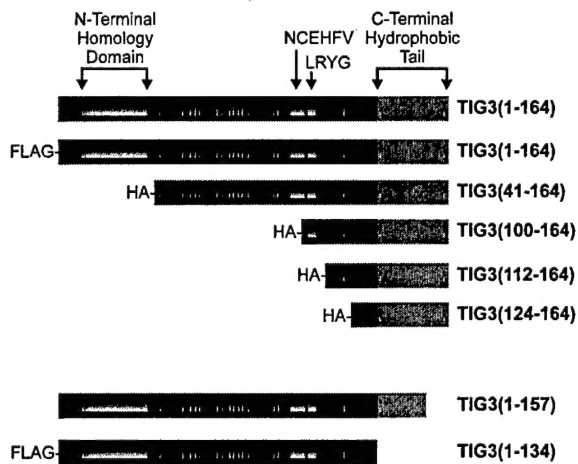
**Fig. 1** Structure of TIG3. The amino terminal segment encodes amino acids 1-134, while the carboxy terminal tail includes amino acids 135-164.

The protein is divided into an amino-terminal domain and a carboxy-terminal hydrophobic domain. We hypothesize that TIG3 subcellular

distribution is controlled by the carboxy-terminal hydrophobic domain and that this serves to anchor

TIG3 to membranes. We further hypothesize that various conserved domains in the amino terminus are required to mediate the growth inhibitory effects. To test this idea we constructed the mutants shown in **Fig. 2**. TIG3<sub>1-164</sub> encodes the full-length protein, while TIG3<sub>1-134</sub> encodes the TIG3 amino-terminus but lacks the carboxy-terminal tail. In the previous report, we described studies showing that TIG3<sub>1-164</sub> efficiently inhibits proliferation, while TIG3<sub>1-134</sub> is minimally effective [2613]. Thus, these studies showed that the carboxyl terminal domain is required for activity. These studies also revealed that TIG3 is localized in the perinuclear region in cells [2613]. These early studies were performed using plasmid-based expression systems that were not very efficient in that transfection would only target 10% of cells in a dish. This plasmid-based system required elaborate selection protocols to quantitate cell kill results [2613]. To circumvent this problem, we subcloned full-length TIG3 (TIG3<sub>1-164</sub>), and all of the other mutants shown in **Fig. 2** into an inducible adenovirus vector, tAd5. This expression system relies on infection with two viruses – one virus that expresses the protein of interest, and a second virus that produces the TET activator protein (Ad5-TA). The TET activator (TA) binds to the promoter in the expression virus to regulate expression. In addition, the TET activator can be inactivated when tetracycline is present in the medium. Thus, expression of TIG3

can be expressed in a regulated manner. This system has two major advantages. First, it delivers protein by infection and, therefore, virtually 100% of the cells can express the protein.



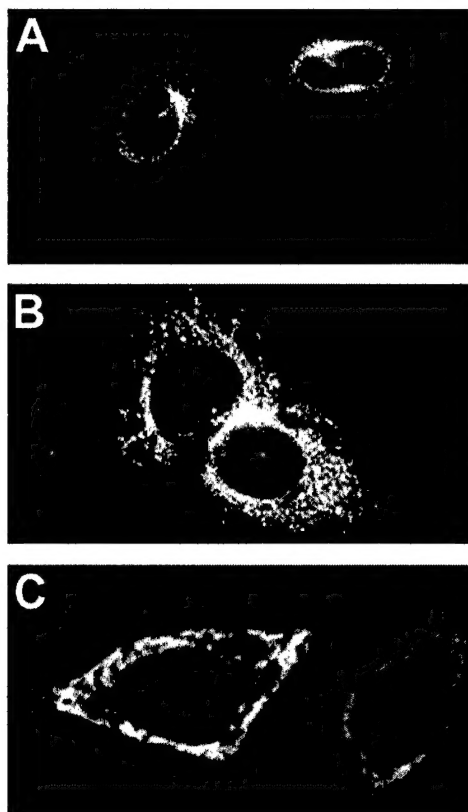
**Fig. 2** TIG3 expression constructs. The various TIG3 encoding segments were synthesized using PCR and then cloned into the tAd5 adenovirus. The resulting recombinant adenoviruses encode the indicated TIG3 mutants. The top construct is full-length TIG3 (TIG3<sub>1-164</sub>). Each of the other constructs has been modified by truncation from the carboxyl or amino-terminal ends. In addition, we have added specific epitope tags to the amino terminal end in most of the constructs (FLAG or HA). Antibodies are available that detect each of these epitopes. The vertical

hatches represent areas of conservation with other members of the TIG3 family of proteins [2613].

Second, the level of TIG3 per cell can be regulated by the level of tetracycline present in the cell culture medium. Additional studies (not shown) indicate that each of the mutant TIG3 proteins encoded by the viruses shown in **Fig. 2** are expressed in virus-infected breast cancer cells. Expression was detected by immunoblot using anti-FLAG, anti-HA, and/or anti-TIG3 antibodies. Each virus was optimized regarding infection ratio etc. so that 100% of the cells are infected and express the TIG3 protein.

TIG3 associates with membranes via its carboxyl terminal hydrophobic domain Using immunohistology, we have demonstrated that full-length TIG3 localizes in the perinuclear region in cells. **Fig. 3** shows this result for CHO cells. A similar subcellular distribution of full-length (TIG3<sub>1-164</sub>) is observed using the adenovirus-based delivery in a variety of cells, including T<sub>47</sub>D breast cancer cells and SK-BR-3 cells. In the most recent funding period, we have tested the other mutants shown in **Fig. 2**. All of the amino-terminal truncation mutants (TIG3<sub>41-164</sub>, TIF3<sub>100-164</sub>, TIG3<sub>112-164</sub>, and TIG3<sub>124-164</sub>) localize with membranes with a distribution similar to full-length TIG3 (TIG3<sub>1-164</sub>). Only, the

C-terminal truncation mutants, TIG3<sub>1-157</sub> and TIG3<sub>1-134</sub> do not localize with membranes - these mutants are present diffusely throughout the cytoplasm.

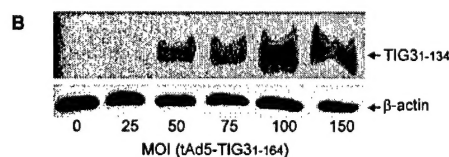
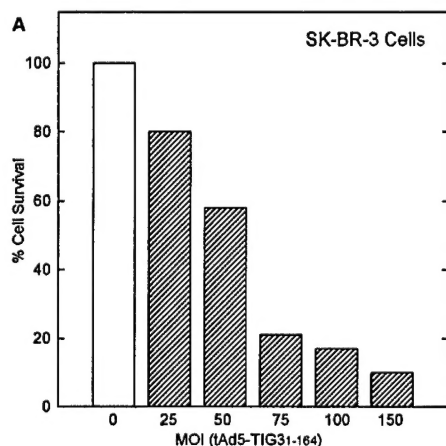


Based on these studies, we conclude that the hydrophobic carboxyl end localizes TIG3 to membranes.

**Fig. 3** Subcellular localization of TIG3<sub>1-164</sub> and TIG3<sub>1-134</sub> in CHO cells. Cells were transfected with expression plasmid encoding TIG3<sub>1-164</sub> (panels A and B) or TIG3<sub>1-134</sub> (panel B). Cells in panel B were additionally treated with cycloheximide {2613}. CHO cells were chosen as an initial test cell since they are easily transfected.

#### Regulation of breast cancer cell proliferation by TIG3

We next evaluated the effects of each mutant on cell proliferation and survival. Cells were plated in 35 mm dishes and allowed to attach overnight and then infected with 0 – 150 MOI of adenovirus. The virus was then removed and incubation was continued for an additional 60 h at which time the cells were harvested and assessed for viability. **Fig. 4A** shows that SK-BR-3 cell number is dramatically reduced by TIG3<sub>1-164</sub> expression. Cell number is 50% reduced at an MOI = 50, and 90% at MOI ≥ 75. **Fig. 4B** shows that the growth suppression is directly correlated with increased TIG3<sub>1-164</sub> expression. In parallel studies, we examined the ability of the TIG3 mutants shown in **Fig. 2** to suppress cell proliferation/survival. Our results are shown in **Table 1**. The growth suppression observed with the TIG3 mutants is compared to that observed when cells are infected with empty adenovirus (tAd5-EV).



**Fig. 4** TIG3 reduces SK-BR-3 cell survival. **A** SK-BR-3 cells were plated, permitted to attach overnight, and then treated with 0 – 150 MOI of tAd5-TIG3<sub>1-164</sub> for 12 h and the virus was removed. After an additional 60 h, the cells were harvested and counted. Percent cell survival at each MOI is calculated by comparison to a group containing an identical amount of tAd5-EV (empty virus). **B** Total cell extracts were prepared from cells treated as above, and then electrophoresed on an 8% gel for immunoblotting with rabbit anti-human TIG3. β-actin was included as a control blot to assure equal loading of protein.

Essentially, all of the mutations result in a significant loss of TIG3 activity. These findings indicate that two domains are essential for TIG3 function – the carboxy-terminal hydrophobic membrane-anchoring domain, and the first 41 amino acids at the amino terminus. We are presently working to identify specific amino acids required for activity.

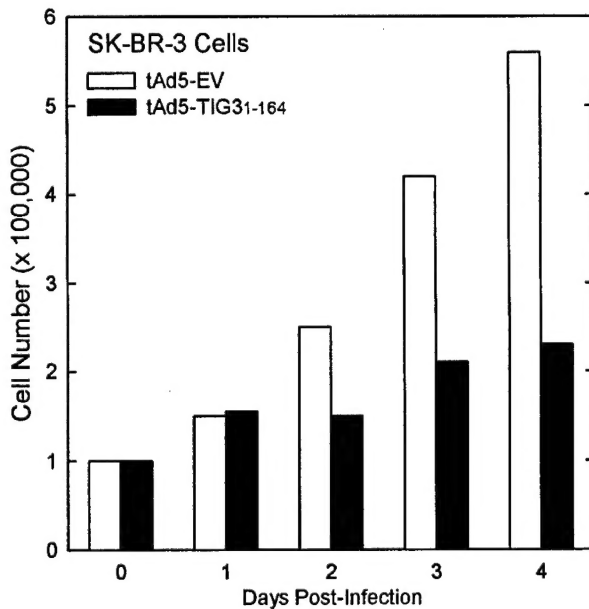


TIG3 Construct	Properties	Relative Cell Number (% of control)
tAd5-EV	Empty vector	100 %
tAd5-TIG3 <sub>1-164</sub>	Full-length TIG3	1%
tAd5-TIG3 <sub>41-164</sub>	Amino-terminal truncation	100%
tAd5-TIG3 <sub>100-164</sub>	Amino-terminal truncation	100%
tAd5-TIG3 <sub>112-164</sub>	Amino-terminal truncation	100%
tAd5-TIG3 <sub>124-164</sub>	Amino-terminal truncation	100%
tAd5-TIG3 <sub>1-157</sub>	Carboxyl-terminal truncation	50%
tAd5-TIG3 <sub>1-134</sub>	Carboxyl-terminal truncation	100%

**Table 1** TIG3 regulation of cell number (100% = no cell death, 0% = no cell survival)

To determine whether the response is reversible, we treated SK-BR-3 cells with 75 MOI of TIG3<sub>1-164</sub>-encoding virus for 12 h. This level of TIG3<sub>1-164</sub> produces optimal suppression of proliferation (see Fig. 4A). The virus was

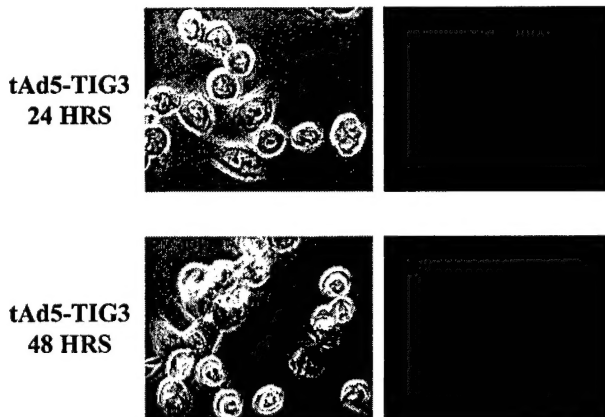
then removed, and cell growth was then continued for the times indicated in Fig. 5. It is clear from this experiment that the cells do not rapidly recover from the treatment with TIG3<sub>1-164</sub>, as growth remains markedly suppressed at day four.



**Fig. 5** SK-BR-3 cells were treated for 12 h with 75 MOI of tAd5-TIG3<sub>1-164</sub> or tAd5-EV (empty vector). The virus was then removed, and incubation was continued for the indicated number of days post-infection. At each time point the cells were harvested and cell number was determined.

suggest that the total number of cells in the culture dish is diminished by TIG3<sub>1-164</sub> treatment. This suggests that in addition to inhibiting cell growth, TIG3 actually reduces the number of cells below the starting level. This suggests that TIG3 actively causes cell death.

TIG3 promotes apoptosis A major goal of this study is to begin to understand the mechanism whereby TIG3 suppresses human breast cancer cell proliferation. The results summarized in Fig. 4A



**Fig. 6** TIG3 expression causes nuclear condensation. SK-BR-3 cells were infected with adenovirus encoding TIG3<sub>1-164</sub> for 12 h. Fresh medium was added and at 24 h and 48 h cells were harvested, fixed (2% paraformaldehyde for 20 min at RT, 100% methanol for 10 min at 4 °C), and stained with 1 µg/ml Hoechst 33258 stain for 10 min at room temperature. Corresponding bright field (left panels) and fluorescent photographs (right panels) are compared. All cells expressed TIG3<sub>1-164</sub> as detected by immunoblot (not shown). Note the compression of many of the nuclei in the TIG3-expressing cells at 48 h. No nuclear compaction was observed in cells expressing the control (empty) adenovirus (not shown).

For this reason, we have examined the hypothesis that TIG3 promotes apoptosis. **Fig. 6** shows an experiment in which SK-BR-3 cells were treated for 24 or 48 h with tAd5-TIG3<sub>1-164</sub>. All cells in the tAd5-TIG3<sub>1-164</sub> group expressed TIG3 as measured by immunohistology (not shown). The left panel in each set shows the bright field image of the cell field. The right panel shows the Hoechst-stained nuclei. It is important to note that the nuclei of the cells in the tAd5-EV group are of normal size (not shown). In contrast, the nuclei in many of the cells in the tAd5-TIG3<sub>1-164</sub>-infected group at 48 h are reduced in size. These images show that the nuclei are compacted in TIG3-expressing cells, a mark of undergoing apoptosis. In addition, preliminary studies suggest that PARP cleavage occurs in TIG3-expressing cells, providing further evidence that TIG3 induces apoptosis.

### Specific Aim 3

- *The TIG3 amino acid sequence/structure reveals no obvious catalytic functional domains, suggesting that TIG3 acts by modulating the function of other proteins. The goal of this specific aim is to identify candidate target proteins using co-immunoprecipitation and affinity chromatography.*

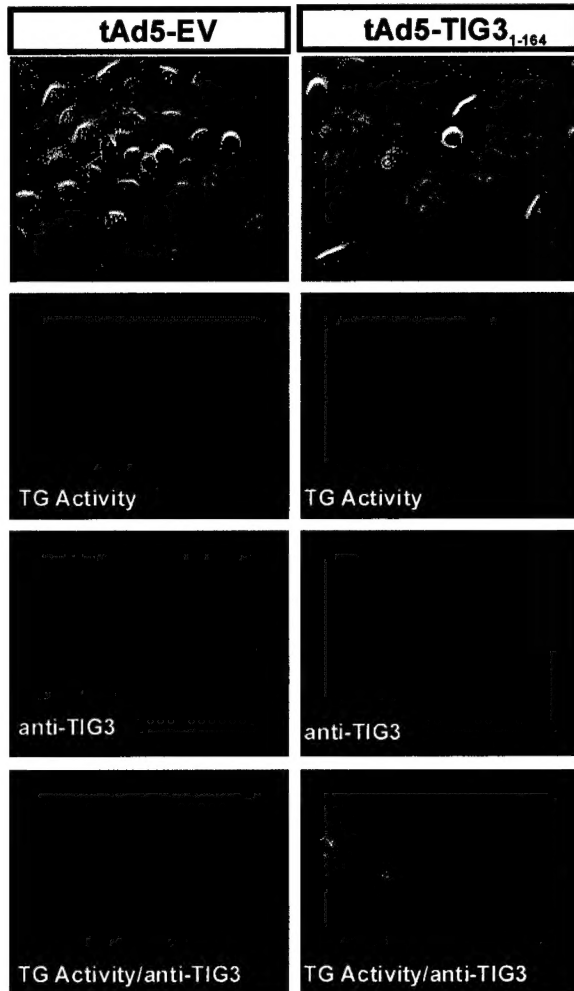
We have proposed that TIG3 binds to target proteins to regulate cell proliferation and survival. A major goal of this study is to identify proteins that interact with TIG3, in order to understand its mechanism of action. We initiated these studies by expressing TIG3 in cells and preparing cell extracts that could be used for TIG3 antibody pull down experiments. However, a major problem was the inability to express enough TIG3 protein to make these biochemical experiments possible. This was due to the use of a plasmid-based expression system, and the fact that TIG3 kills cells very efficiently and limits the number of TIG3-expressing cells that can be obtained. To circumvent these difficulties we switched from the plasmid-based to an adenovirus-based expression system. The use of this system has solved the problem of minimal protein expression. In addition, it has solved the problem of cell viability, since we are using an inducible expression system that permits TIG3 expression to be turned on and off.

The major breakthrough in this work came when we transfected a normal, non-transformed, cell type. Since TIG3 is expressed at normal levels in non-transformed cells, we hypothesized that putative TIG3 targets may also be expressed at normal levels. We used primary cultures of normal human keratinocytes, the major normal cell type of epidermis, as our test cell. These cells were infected with TIG3-encoding, tetracycline-inducible adenovirus. The remarkable finding from these studies is that TIG3 interacts with transglutaminase.

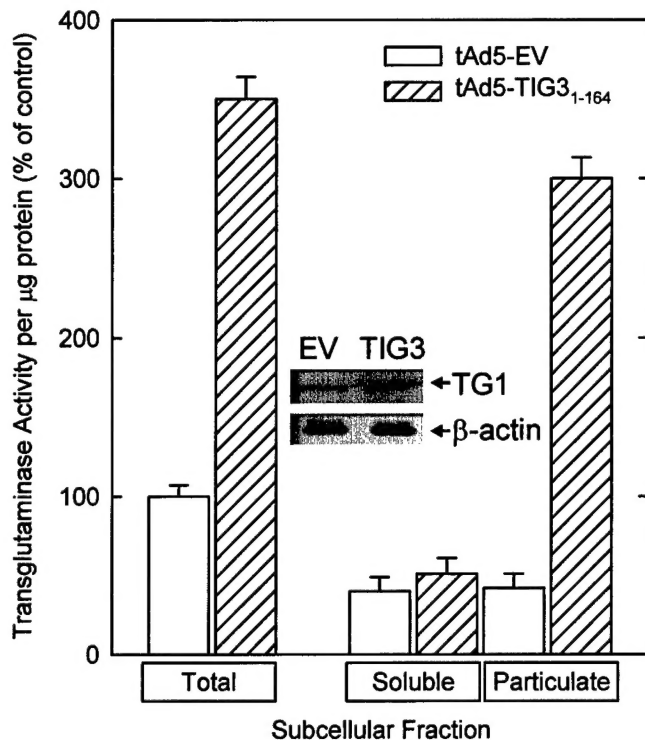
TIG3 activates transglutaminase Transglutaminases (TGs) are a family of enzymes that assemble differentiation- and cell death-associated structures, inside cells. To determine whether TIG3 influences TG activity, cells were treated with tAd5-EV or tAd5-TIG3<sub>1-164</sub> for 48 h. During the last 4 h of treatment, the medium was supplemented with 100  $\mu$ M FC, a cell-permeable fluorescent transglutaminase substrate. **Fig. 7** shows that FC incorporation (green) is greatly increased in TIG3 expressing (red) cells. As a second method of detecting TIG3-dependent transglutaminase activity, **Fig. 8** shows that total transglutaminase activity, measured by monitoring <sup>3</sup>H-putrescine incorporation, is increased 3-fold by TIG3<sub>1-164</sub>. Separation of lysate into soluble and particulate fractions reveals that most of the increase in transglutaminase activity is observed in the particulate (membrane) fraction, suggesting that membrane-associated type I transglutaminase is activated.



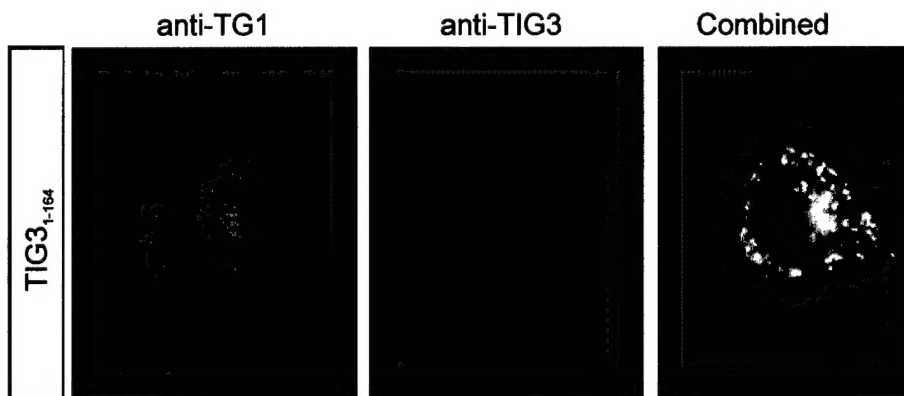
Although the increase in TG1 activity could be due to increased expression of TG1, the immunoblot of total extract prepared from tAd5-EV and tAd5-TIG3<sub>1-164</sub>-infected cells (insert) shows that TG1 levels are not visibly altered by TIG3 treatment. Consistent with activating a membrane-associated target, a cell fractionation study indicated that TIG3 is associated with the membrane fraction (not shown). The above results suggest that TIG3 and TG1 co-localize. As an additional method of monitoring this localization, cells were infected with TIG3<sub>1-164</sub> and, after 48 h, the cells were immunostained with anti-TIG3 (red) and anti-TG1 (green). The epifluorescent images, **Fig. 9**, show that TIG3 and TG1 accumulate in punctate aggregates. The yellow color in the combined image shows that TIG3 and TG1 co-localize in these structures, further suggesting that the two proteins are in close proximity.



**Fig. 7** TIG3 overexpression is associated with increased transglutaminase activity. **A** Keratinocytes, grown on coverslips, were infected for 12 h with 20 MOI of either tAd5-EV or tAd5-TIG3<sub>1-164</sub> encoding adenovirus and 5 MOI of Ad5-TA.. At 48 h post-infection, the cells were pulsed for 4 h with 100  $\mu$ M fluorescein cadaverin (FC, green). The cells were then fixed and stained with rabbit anti-TIG3 and Cy3-conjugated anti-rabbit IgG (red) (7), and visualized by fluorescent light microscopy. The combined image (yellow) indicates co-localization of TIG3 and FC incorporation.



**Fig. 8** Transglutaminase activity was measured *in vitro* by monitoring <sup>3</sup>H-putrescine incorporation. Cells, growing in 50 cm<sup>2</sup> dishes, were infected with either tAd5-EV or tAd5-TIG3<sub>1-164</sub> for 12 h as in panel A. After 48 h whole cell, and particulate and soluble fractions, were isolated and assayed for transglutaminase activity. The open bars indicate activity in cells infected with tAd5-EV, and the hatched bars activity in TIG3<sub>1-164</sub>-expressing cells. The error bars represent standard errors of the mean from three experiments. The insert depicts an immunoblot showing that TG1 level is not significantly altered in TIG3-producing cells. A β-actin immunoblot is included as a protein loading control.



**Fig. 9** TG1 and TIG3 co-localization. Keratinocytes were grown on coverslips and then infected for 48 h with tAd5-TIG3<sub>1-164</sub>. The cells were then fixed, permeabilized and stained with rabbit anti-TIG3 (1:100)/Cy3-conjugated goat anti-rabbit IgG (1:1000, Sigma), and mouse anti-transglutaminase type 1

(1:50)/Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000). The red (anti-TIG3) and green (anti-TG1) fluorescence was detected by using a Nikon epi-fluorescent microscope.

**TIG3 and TG1 co-precipitate** The above studies suggest that TIG3 and TG1 co-localize in cells, and that TG1 is active in the presence of TIG3. A recent experiment clearly indicates that TG1 and TIG3 interact. Cells were infected with adenoviruses expressing HA-TIG3 and FLAG-tagged TG1. After 48 h, extracts were prepared for immunoprecipitation. Precipitation with anti-HA pulls down FLAG-TG1, while precipitation with anti-FLAG-TG1 pulls down HA-TIG3 (not shown). These

findings will be the topic of a subsequent publication. In addition, we are beginning to transfer this knowledge back to the breast cancer system. Breast cancer cells also produce a form of transglutaminase, type II transglutaminase, that is involved in cell apoptosis. Our present thrust is to determine whether TIG3 kills breast cancer cells by targeting and activating this enzyme.

## KEY RESEARCH ACCOMPLISHMENTS

### ***Previous Report Periods***

- We have constructed plasmid-based TIG3 expression systems and used these to express TIG3 in cells (*Specific Aims 1 and 2*)
- We have shown that TIG3 assumes a perinuclear location in cells, and that a mutant lacking the carboxyl terminus does not assume this location (*Specific Aim 1*)
- We have demonstrated that the TIG3 carboxy-terminal hydrophobic domain is required for appropriate subcellular localization (*Specific Aim 1*)
- Eliminating the TIG3 carboxy-terminal tail reduces the ability of TIG3 to kill cells (*Specific Aim 2*)
- Adenovirus expression systems have been constructed that permit more efficient studies of cell killing and permits efficient production of TIG3 in cells for biochemical studies (*Specific Aims 1, 2 and 3*). This virus produces high-level expression of TIG3 in MCF7 cells.
- TIG3 kills breast cancer cell lines (e.g., T<sub>47</sub>D, SK-BR-3) (*Specific Aim 2*)
- We have extended our results to show that TIG3 expression kills SK-BR-3 breast cancer cells and our findings suggest that TIG3 promotes breast cancer cell apoptosis and activation of transglutaminase activity (*Specific Aim 1*)
- We have constructed a number of TIG3 mutants, and identified the carboxyl-terminal hydrophobic domain and the N-terminal 41 amino acids as essential for function. We are presently identifying specific amino acids that are required for activity (*Specific Aims 1 and 2*)

### ***Present Report Period***

- We have identified type I transglutaminase as a target of TIG3 in eukaryotic cells.
- We have shown that TIG3 activates apoptosis in some cells and increases transglutaminase activity in other cells - both are associated with different cell death pathways.

## REPORTABLE OUTCOMES

- We have two manuscripts published, and one additional manuscript will be submitted later this year.
- Mike Sturniolo, a graduate student, reported on the effects of TIG3 and the International Transglutaminase Conference in Ferrara Italy in September 2002.

- Anne Deucher, an M.D./Ph.D. student has completed her thesis. Fifty percent of the thesis focused on TIG3 function.
- Mike Sturniolo's entire Ph.D. thesis, which is 1/2 completed, will focus exclusively on TIG3. He expects to complete his Ph.D. degree in May 2004.
- Shervin Dashti, an M.D./Ph.D. student, who completed a number of the early TIG3 studies and will be a co-author on several TIG3 papers, recently completed his Ph.D.
- A post-doctoral associate, Ann-Marie Broome obtained her first molecular training on this project. She is a coauthor on one of the manuscripts resulting from this study.

## CONCLUSIONS

We consider this work to be very important from the point-of-view of future breast cancer therapeutics. Our studies completed to date clearly show that TIG3 inhibits the breast cancer cell proliferation. We also suspect that TIG3 has the ability to kill (cause apoptosis) breast cancer cells independent of its effects on cell proliferation. We strongly suspect, based on data generated during the most recent funding period, that TIG3 acts by activating an intracellular crosslinking enzymes called transglutaminase. We plan to resubmit this proposal for additional support for breast cancer funding – specifically to translate this new information back to the breast cancer cell system.

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